Table S1: Yeast strains used in this study

Strain	Genotype	Source
PJ69-4A	MATa *trp1-Δ901 leu2-3,112 901 ura3-52 his3-Δ200 gal4Δ gal8Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	(James *et al.*, 1996)
SEY6210	MATα *his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9 GAL*	(Robinson *et al.*, 1988)
TKYM22	SEY6210 *OM45-GFP::TRP1*	(Kanki and Klionsky, 2008)
TKYM25	SEY6210 *atg1Δ::KanMX6 OM45-GFP::TRP1*	(Kanki *et al.*, 2009)
TKYM140	SEY6210 *atg32Δ::LEU2 OM45-GFP::TRP1*	This study
TKYM165	SEY6210 *atg32Δ::HIS5 S.p.*	This study
TKYM218	SEY6210 *atg11Δ::LEU2 atg32Δ::HIS5 S.p.*	This study
TKYM236	SEY6210 *pho8Δ::HIS5 S.p. pho13Δ::LEU2*	This study
TKYM242	SEY6210 *hog1Δ::HIS5 S.p.*	This study
TKYM243	SEY6210 *pbs2Δ::HIS5 S.p.*	This study
TKYM248	SEY6210 *pho8Δ::HIS5 S.p. pho13Δ::LEU2 hog1Δ::URA3*	This study
TKYM249	SEY6210 *pho8Δ::HIS5 S.p. pho13Δ::LEU2 pbs2Δ::URA3*	This study
TKYM250	SEY6210 *hog1Δ::HIS5 S.p. OM45-GFP::TRP1*	This study
TKYM251	SEY6210 *pbs2Δ::HIS5 S.p. OM45-GFP::TRP1*	This study
TKYM254	SEY6210 *hog1Δ::HIS5 S.p. PEX14-GFP:: KanMX6*	This study
TKYM255	SEY6210 *pbs2Δ::HIS5 S.p. PEX14-GFP:: KanMX6*	This study
TKYM256	SEY6210 *pho8Δ::HIS5 S.p. pho13Δ::LEU2 atg1Δ::URA3*	This study

TKYM280	BY4742 *OM45-GFP::His3MX6*	This study
TKYM281	BY4742 *ssk2Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM282	BY4742 *ssk22Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM283	BY4742 *ste11Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM284	BY4742 *ssk1Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM285	BY4742 *msb2Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM286	BY4742 *sho1Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM287	BY4742 *atg1Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM288	SEY6210 *atg1Δ::KanMX6* *atg32Δ::LEU*	This study
TKYM290	SEY6210 *hog1Δ::HIS5 S.p.* *atg1Δ::LEU2*	This study
TKYM291	SEY6210 *pbs2Δ::HIS5 S.p.* *atg1Δ::LEU2*	This study
TKYM292	BY4742 *hog1Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
TKYM293	BY4742 *pbs2Δ::KanMX6* *OM45-GFP::HIS3MX6*	This study
WHY1	SEY6210 *atg1Δ::HIS5 S.p.*	(Shintani *et al.*, 2002)
YTS147	SEY6210 *atg11Δ::LEU2*	(Kanki and Klionsky, 2008)

References

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Robinson, J.S., Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol *8*, 4936-4948.

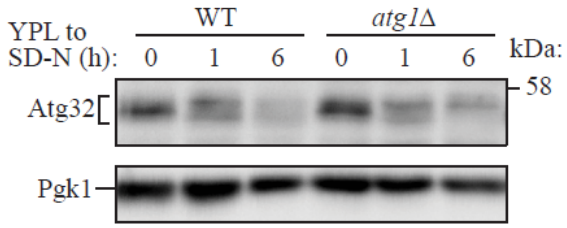
Kanki, T., and Klionsky, D.J. (2008). Mitophagy in yeast occurs through a selective mechanism. J Biol

Chem *283*, 32386-32393.

Kanki, T., Wang, K., Baba, M., Bartholomew, C.R., Lynch-Day, M.A., Du, Z., Geng, J., Mao, K., Yang, Z., Yen, W.L., and Klionsky, D.J. (2009). A genomic screen for yeast mutants defective in selective mitochondria autophagy. Mol Biol Cell *20*, 4730-4738.

Shintani, T., Huang, W.P., Stromhaug, P.E., and Klionsky, D.J. (2002). Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Dev Cell *3*, 825-837.

A



B

```
  1 MVLEYQQREGKGSSSKSMPPDSSSTTIHTCSEAQTGEDKGLLDPHLSVLELLSKTGHSPS  60

                                                        114  119
 61 PMGQNLVTSIDISGNHNVNDSISGSWQAIQPLDLGASFIPERCSSQTTNGSILSSSDTSE 120

121 EEQELLQAPAADIINIIKQGQEGANVVSPSHPFKQLQKIISLPLPGKEKTPFNEQDDDGD 180

181 EDEAFEEDSVTITKSLTSST.........................ILALGYILL*    529
                                                          stop
```

C

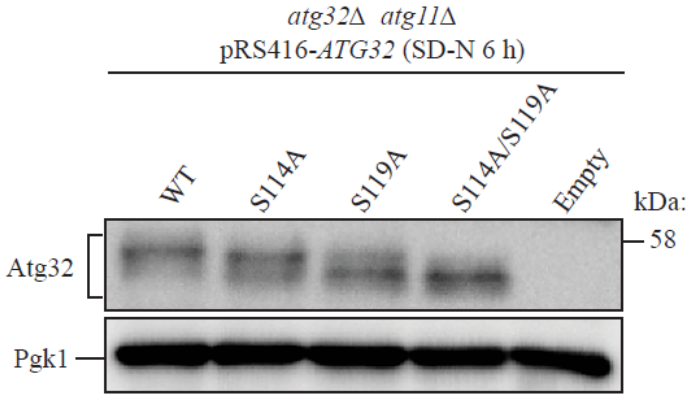


Figure S1

(A) Wild-type (WT) or *atg1Δ* strains were cultured in YPL medium until the mid-log growth phase and then shifted to SD-N medium for 0, 1 and 6 hours. The amount and the modification of endogenous Atg32 were observed by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies. (B) Amino acid sequence of Atg32. Ser or Thr residues, which we screened as candidate phosphorylation sites on Atg32, are denoted in red. (C) To clarify the change in molecular weight of Atg32 mutants, samples from the same experiment shown in Figure 2C (SD-N 6 h) were evaluated by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies.

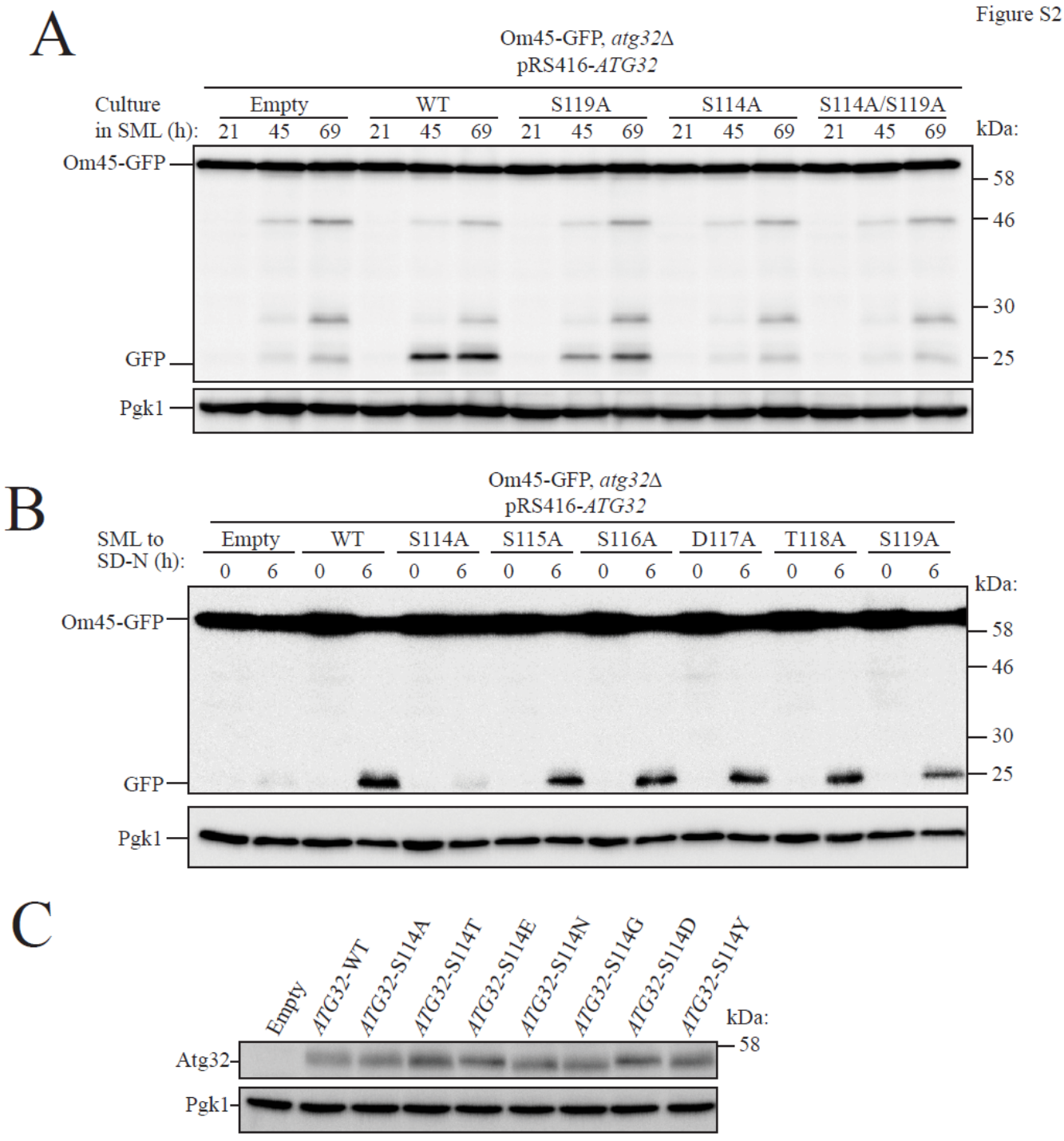


Figure S2

(A) Strains of *atg32Δ* expressing Om45-GFP were transformed with the indicated Atg32 mutants expressing vectors. Cells were cultured in SML medium for a maximum of 69 hours to allow cellular growth to the post-logarithmic phase, and cells were collected at the indicated periods. GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies. (B)

Strains of *atg32Δ* expressing Om45-GFP were transformed with the indicated Atg32 mutant-expressing vectors. Cells were cultured in SML medium until the mid-log growth phase and then shifted to SD-N for 6 hours. GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies. (C) The *atg32Δ* strain was transformed with the indicated Atg32 mutant-expressing vectors. Cells were cultured in SML medium until the mid-log growth phase and the expression level of Atg32 mutants was observed by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies.

Figure S3

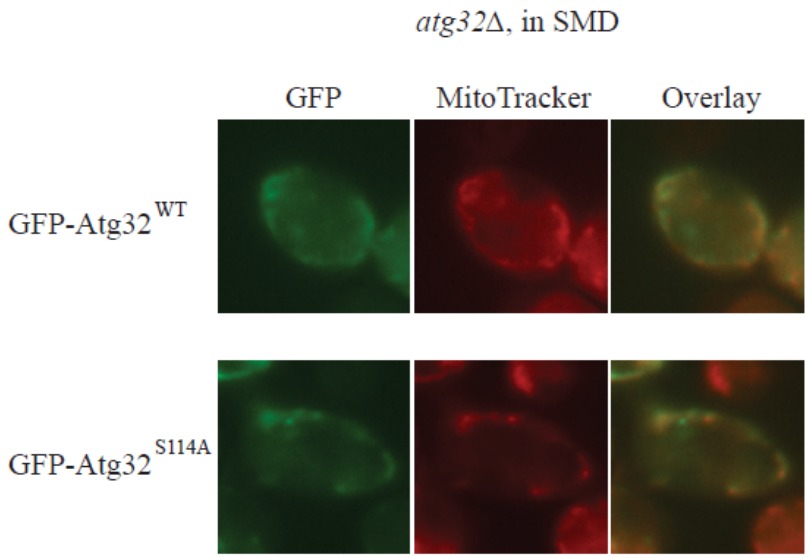


Figure S3

The *atg32Δ* strain transformed with a plasmid expressing GFP-tagged $Atg32^{WT}$ or $Atg32^{S114A}$ under the control of the *CUP1* promoter was cultured in SMD medium until the mid-log growth phase. Cells were labeled with the mitochondrial marker MitoTracker Red and analyzed by fluorescence microscopy.

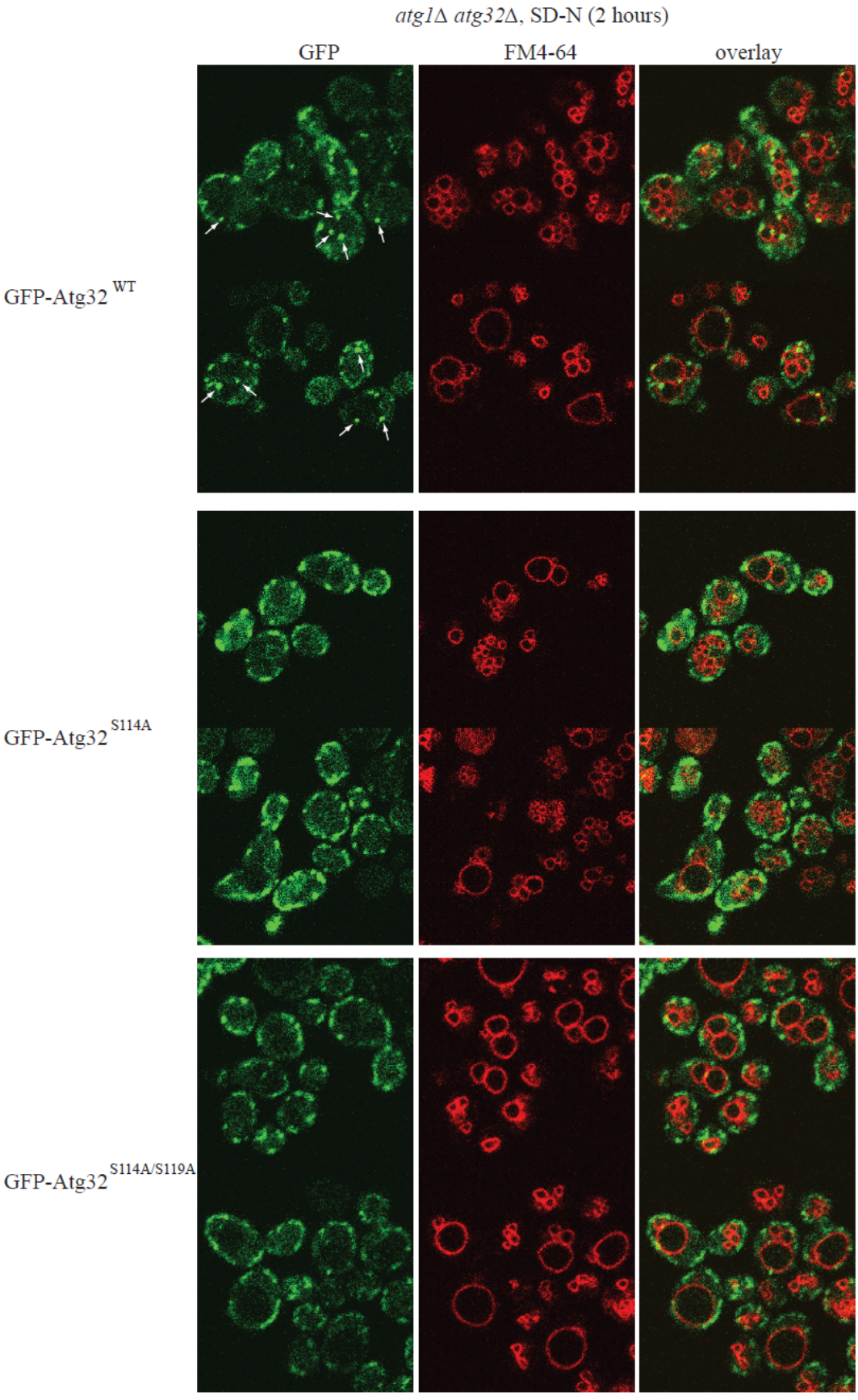

*atg1*Δ *atg32*Δ, SD-N (2 hours)
GFP
FM4-64
overlay
GFP-Atg32 WT
GFP-Atg32 S114A
GFP-Atg32 S114A/S119A

Figure S4

Atg1Δ and *atg32Δ* double knockout strains transformed with a plasmid expressing GFP-tagged $Atg32^{WT}$, $Atg32^{S114A}$, or $Atg32^{S114A/S119A}$ under the control of the *CUP1* promoter were cultured in SMD medium until the mid-log growth phase, and then shifted to starvation medium (SD-N) for 2 hours. Cells were labeled with the vacuolar marker FM4-64 and analyzed by confocal laser microscopy.

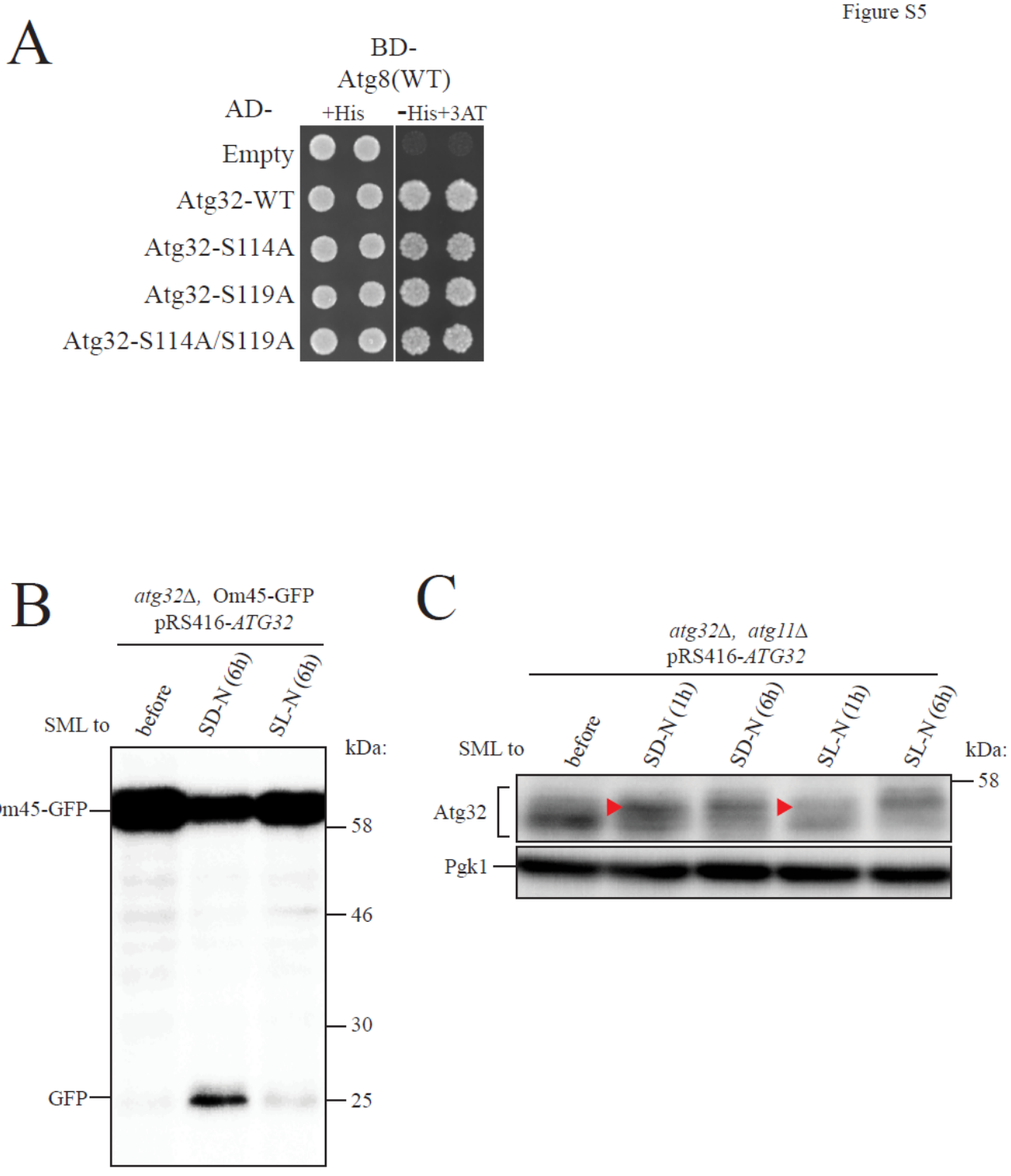
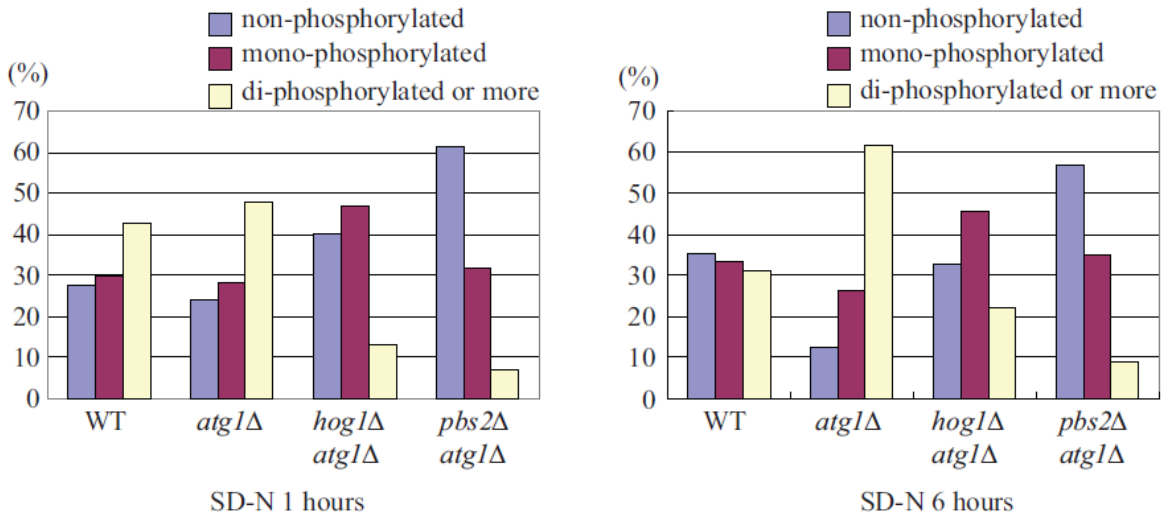


Figure S5

A) Yeast two-hybrid analysis between Atg8 and Atg32 mutants. The PJ69–4A strain was transformed with pGAD and pGBD plasmids, which can express the indicated proteins. Cells were grown on +His plate or –His plate supplemented with 6 mM 3-aminotriazole (3AT) and grown at 30°C for 4 days. (B) Strains of *atg32Δ* expressing Om45-GFP were transformed with wild-type Atg32-expressing vectors.

Cells were cultured in SML medium until the mid-log growth phase and then shifted to SD-N or SL-N for 6 hours. GFP processing was monitored by immunoblotting with anti-GFP antibodies. (C) The *atg32Δ* and *atg11Δ* double knockout strains transformed with wild-type Atg32-expressing vector were cultured in SML medium until the mid-log growth phase, and then shifted to SD-N or SL-N for 1 and 6 hours. The modification of Atg32 mutants was monitored by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies.

A


non-phosphorylated
mono-phosphorylated
di-phosphorylated or more
(%)
70
60
50
40
30
20
10
0
WT
atg1Δ
hog1Δ atg1Δ
pbs2Δ atg1Δ
SD-N 1 hours
SD-N 6 hours

B

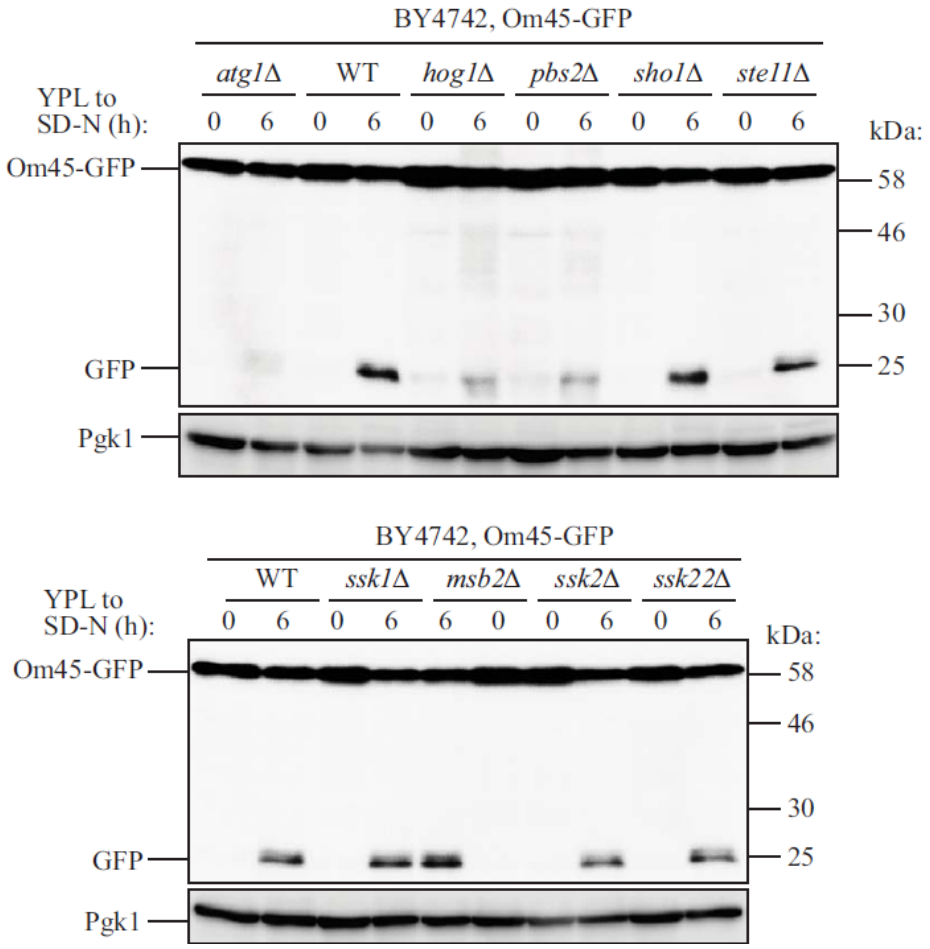

BY4742, Om45-GFP
atg1Δ
WT
hog1Δ
pbs2Δ
sho1Δ
ste11Δ
YPL to SD-N (h):
0 6 0 6 0 6 0 6 0 6 0 6
kDa:
Om45-GFP
GFP
Pgk1
58
46
30
25
BY4742, Om45-GFP
WT
ssk1Δ
msb2Δ
ssk2Δ
ssk22Δ
YPL to SD-N (h):
0 6 0 6 6 0 0 6 0 6
kDa:
Om45-GFP
GFP
Pgk1
58
46
30
25

Figure S6

(A) The signal intensity of each phosphorylated and non-phosphorylated bands of an immunoblot image shown in Figure 4A were quantified using Image Gauge software (Fujifilm). (B) Strains deleted for the indicated genes and expressing Om45-GFP were cultured in YPL medium until the mid-log growth phase and then shifted to SD-N for 6 hours. GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies.

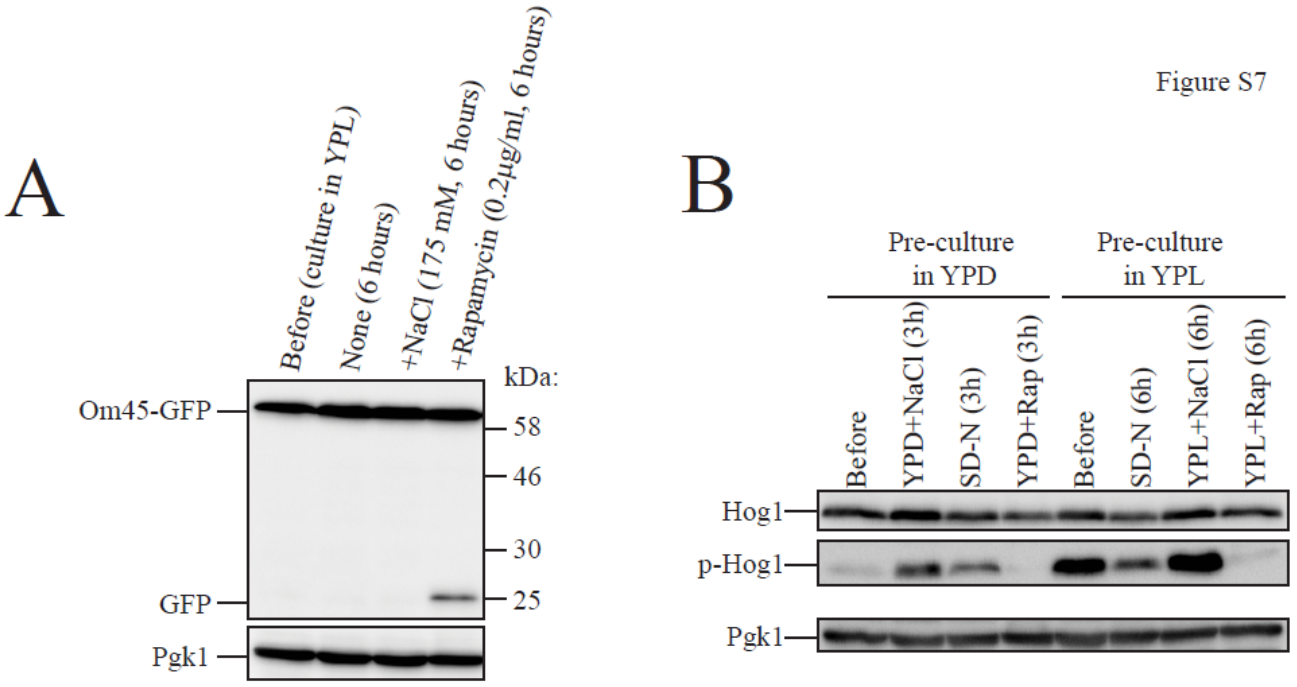


Figure S7

(A) Wild-type cells expressing Om45-GFP were cultured in YPL medium until the mid-log growth phase and then NaCl (for osmotic stress) or rapamycin (as a positive control for mitophagy) was added. After 6 hours, GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies. (B) Wild-type cells were cultured in YPD or YPL until the mid-log growth phase and NaCl (175mM) or 0.2 μg/ml rapamycin was added or shifted to SD-N for the indicated periods. Total Hog1 expression, phosphorylated Hog1, and Pgk1 (loading control) were observed by immunoblotting.

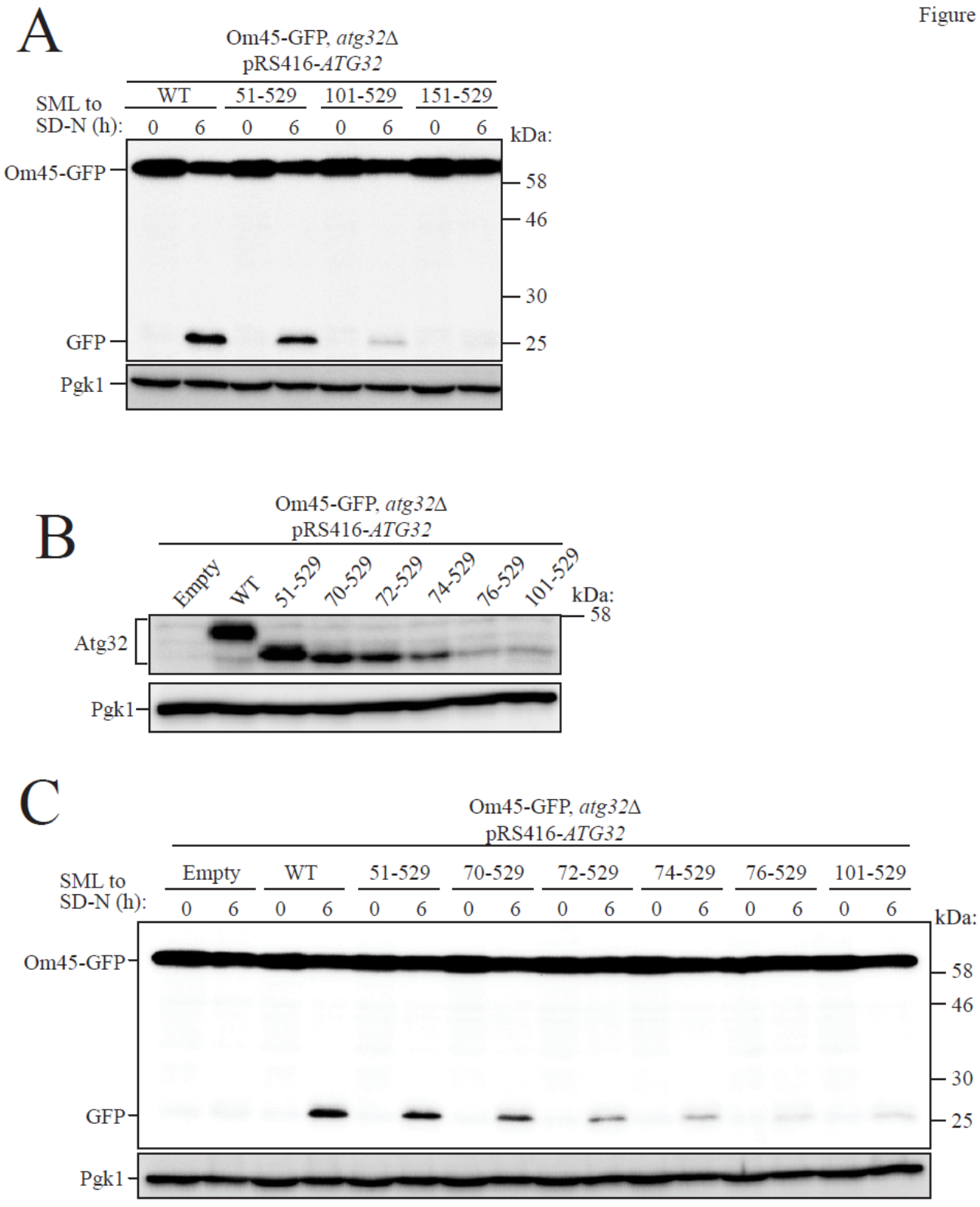


Figure S8

(A, C) Strains of *atg32Δ* expressing Om45-GFP were transformed with the indicated Atg32

mutant-expressing vectors. Cells were cultured in SML medium until the mid-log growth phase and then shifted to SD-N for 6 hours. GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies. (B) The same cells shown in panel C were cultured in SML medium until the mid-log growth phase and the expression level of Atg32 mutants was observed by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies.

Figure S9

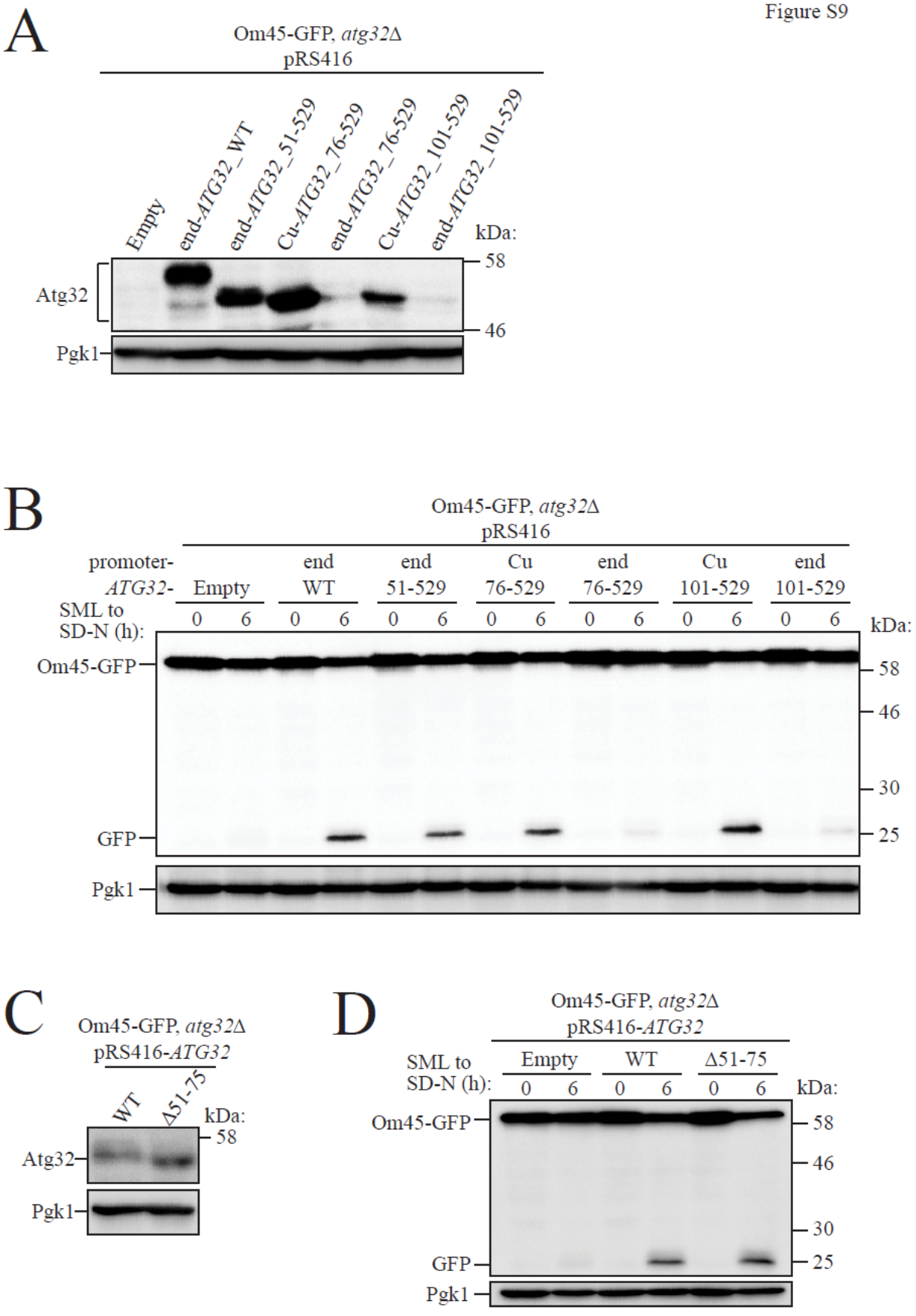

A
Om45-GFP, atg32Δ
pRS416
Empty
end-ATG32_WT
end-ATG32_51-529
Cu-ATG32_76-529
end-ATG32_76-529
Cu-ATG32_101-529
end-ATG32_101-529
kDa:
58
46
Atg32
Pgk1
B
Om45-GFP, atg32Δ
pRS416
promoter-ATG32-
Empty
end WT
end 51-529
Cu 76-529
end 76-529
Cu 101-529
end 101-529
SML to SD-N (h):
0 6 0 6 0 6 0 6 0 6 0 6 0 6
kDa:
Om45-GFP
GFP
Pgk1
58
46
30
25
C
Om45-GFP, atg32Δ
pRS416-ATG32
WT
Δ51-75
kDa:
58
Atg32
Pgk1
D
Om45-GFP, atg32Δ
pRS416-ATG32
SML to SD-N (h):
Empty
WT
Δ51-75
0 6 0 6 0 6
kDa:
Om45-GFP
GFP
Pgk1
58
46
30
25

Figure S9

(A, C) Strains of *atg32Δ* expressing Om45-GFP were transformed with plasmids expressing Atg32 mutants under the endogenous *ATG32* promoter (pRS416-end-*ATG32*_WT, pRS416-end-*ATG32*_51-529, pRS416-end-*ATG32*_76-529, pRS416-end-*ATG32*_101-529, or pRS416-end-*ATG32*_Δ51-75) or under the *CUP1* promoter (pRS416-Cu-*ATG32*_76-529 or pRS416-Cu-*ATG32*_101-529). Cells were cultured in SML medium until the mid-log growth phase and the expression level of Atg32 mutants was observed by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies. (B, D) The same cells shown in panels A and C were cultured in SML until the mid-log growth phase and then shifted to SD-N for 6 hours. GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies.